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Attorney's Docket No.: 12071-011002

REMARKS

Claims 54, 57-59, and 61-106 are pending in the application, claims 55, 56, and 60 having been canceled and claims 68-106 having been added by the present amendment.

The amendment of claim 54 is supported by the specification at, for example, page 5, lines 5-6 and at page 11, lines 2-9. Claims 57-59 and 62-67 have been amended for clarity (to, *e.g.*, correct inadvertent grammatical errors and provide appropriate antecedent basis for the claim terms). Claim 61 has been amended to read as an independent claim. New claims 68, 77, 92, and 99 are supported by the specification at, for example, page 1, lines 9-16. New claims 69, 78, 93, and 100 are supported by the specification at, for example, page 4, lines 29-30. New claims 70-73, 79, 80, 84, 94, 95, 101 and 102 are supported by the specification at, for example, page 14, lines 17-19. New claims 74, 75, 90, 91, 105, and 106 are supported by, for example, original claims 3 and 9. New claims 76 and 88 are supported by the specification at, for example, page 24, lines 3-8. New claims 81, 82, 83, 103, and 104 are supported by the specification at, for example, page 15, lines 18-22. New claims 87 and 89 are supported by the specification at, for example, page 5, lines 7-13. New claims 96-98 are supported throughout the specification at, for example, page 19, lines 3-8 and 24-31, page 30, lines 16-18, page 31, lines 4-9 and the Examples at pages 34-52. No new matter has been added.

Claim Objections

Claim 66 was objected to as being in an improper form. A multiply dependent claim should refer to other claims in the alternative only, but claim 66 depended from claim 64 and claims 55-62 (Office Action at page 2).

Claim 66 has been amended to refer to other claims in the alternative only. Therefore, this objection may now be withdrawn.

Claims 57 and 60 were objected to because they recited a variety of abbreviations that were not spelled out at their first appearance in the claims (Office Action at page 2).

Claim 60 has been cancelled, and there is no apparent alternative to the terms used in claim 57. Claim 57 includes the terms M1, M2, P1, P2, and PA, which are used routinely by those of ordinary skill in the art. Upon investigation, Applicants' representative could find no alternative names for these antigens, *i.e.*, the terms used do not appear to be abbreviations. The

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Examiner's attention is directed to the attached pages from *Virology*, Lippincott-Raven, Philadelphia, Fields *et al.*, eds., 3rd Edition, 1996 (pages 90, 1358, and 1366; Exhibit A), where the terms M1, M2, PB1, PB2, and PA are introduced, as if complete. Accordingly, this ground for rejection should be withdrawn.

Claim 58 was objected to for reciting "pET65MP/NP-B" and "pET65M/NP/D" as if they were, themselves, proteins. The Examiner is correct in supposing that these terms represent plasmids that encode fusion proteins. Claim 58 has been amended to make this clear. Thus, the objection may now be withdrawn.

Applicants' Declaration

The declaration filed on May 24, 2000, under 37 C.F.R. § 1.131 was found ineffective to overcome Suzue *et al.* (*J. Immunol.* 156:873-879, 1996; herein, "Suzue"). The Examiner states, however, that a declaration stating "that the work was done in the USA, a WTO country or a NAFTA country" and including "the same evidence and arguments present in the instant declaration would be sufficient to overcome the reference" (Office Action at page 3).

The declaration filed herewith is identical to the declaration filed on May 24, 2000, except that Applicants also declare that "[t]he work described in the Exhibit was conducted in the United States of America or Canada" (see ¶ 6; Exhibit B). The declaration should, therefore, effectively remove not only Suzue, but also Roman *et al.* (*Immunol.* 88:487-492, 1996; herein, "Roman"). Roman was published after Suzue (*i.e.*, after January 1, 1996).

As neither Suzue nor Roman can be applied against claims in the present application, the rejections based on these references (the rejection of claims 54, 55, 59, and 61-67 for alleged lack of novelty in view of Suzue and the rejection of claims 56-58 for alleged obviousness over Roman and Suzue) must be withdrawn.

New Grounds for Rejection

The Examiner states (Office Action at page 6):

[a]pplicant has replaced the previously pending claims with claims limited to fusion proteins, but not limited in the nature of the stress protein. These amendments required a new search and the application of new art.

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The present action cannot properly be made final. The new ground of rejection is neither necessitated by Applicants' amendment nor based on information submitted in an information disclosure statement. MPEP at 706.07(a). Applicants' amendment limited the compositions claimed to fusion proteins, but these compositions have been before the Examiner since prosecution began. Applicants elected to prosecute the claims of Group I, which included the following claim (claim 5):

5. The vaccine of Claim 1 wherein the antigen and the stress protein are linked as a fusion protein.

Thus, the Examiner could have, and should have, searched for art concerning fusion proteins earlier. Nothing in Applicants' amendment necessitated a new search. Accordingly, the Examiner is asked to reconsider and withdraw the "finality" of the present Office Action (and enter the amended claims shown above).

35 U.S.C. § 102(b)

Claims 54, 55, 58, and 61-67 are rejected as being anticipated by Young (WO 94/29459; herein, "Young"). In characterizing Young, the Examiner states (Office Action at page 6; emphasis added):

- * Young discloses fusion proteins of microbial stress proteins and an antigen of interest
- * Young sets forth that fusion proteins can be made between a stress protein and any antigen
- * Antigens of viral pathogens, bacteria, or cancer cells are specifically contemplated
- * The fusion proteins are administered to mice in a pharmacologically acceptable carrier or diluent, phosphate buffered saline

This ground for rejection should be withdrawn in view of the present amendment. As the Examiner knows, "[t]o anticipate a claim, a reference must disclose every element of the challenged claim and enable one skilled in the art to make the anticipating subject matter." *PPG Industries, Inc. v. Guardian Industries Corp.* 75 F.3d 1558 (Fed. Cir. 1996). Young does not "disclose every element" of the present claims. Each of the present independent claims is limited

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to a fusion protein (or compositions containing it, or methods of using it) that includes a stress protein, or a portion thereof, and a particular antigen or an antigenic portion thereof. For example, claim 54 is limited to a fusion protein comprising an antigen of the influenza virus; ~~and claim 69 is limited to a fusion protein comprising a human papillomavirus (HPV) antigen;~~ ~~claim 79 is limited to a fusion protein comprising particular tumor-associated antigens; and claim 88 is limited to a fusion protein comprising particular allergens.~~ Young does not disclose an antigen of the influenza virus, ~~an HPV antigen, or the particular tumor-associated antigens and allergens covered by the present claims.~~ Therefore, Young cannot anticipate the present claims.

Young's disclosure of generic fusion proteins is not enough. While the earlier disclosure of a species defeats a claim to the genus encompassing it, the opposite is not true. *In re Gosteli*, 872 F.2d 1008 (Fed. Cir. 1989). An earlier disclosure of the genus does not anticipate each and every species within it. For example, in *Corning Glass Works v. Sumitomo Electric U.S.A.*, the patent at issue claimed germania as a dopant for use in an optical waveguide fiber. Although the prior art disclosed waveguide fibers with doped cores, and suggested titania as the dopant, the court found the claims covering the use of germania were not anticipated. The court noted that the prior art did not expressly disclose germania, nor did it exclude it. 868 F.2d 1251 (Fed. Cir. 1989).

The facts of the present case are entirely consistent with those in *Corning Glass*. Here, Applicants claim fusion proteins containing specific antigens that were neither disclosed in, nor excluded by, the prior art. The rejection for anticipation should therefore be withdrawn.

35 U.S.C. § 103

Claims 56-58 and 60 are rejected as being obvious over Young in view of Srivastava (U.S. Patent No. 5,837,251; herein, "the '251 patent"). Claim 56 has been canceled, but the limitation of that claim (that the antigen is an antigen of the influenza virus) has been incorporated into amended claim 54. Claim 60 has also been canceled, but the limitations of that claim have been incorporated into other claims as well (see, e.g., new claims 69, 70, and 79).

As a preliminary matter, Applicants note that claims 61-67 are not rejected as being obvious. For the reasons described above, these claims cannot, as a matter of law, be rightfully

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rejected for lack of novelty. Accordingly, at least claims 61-67 are now in condition for allowance (claim 61 has been rewritten as an independent claim), which action is respectfully requested.

In describing the basis for the obviousness rejection of claims 56-58 and 60, the Examiner reiterates her characterization of Young (Office Action at pages 7-8), and then states that the '251 patent "discloses complexes of hsp proteins from the hsp60, hsp70 and hsp90 families in complex with antigens such as tumor antigens or influenza antigens" (Office Action at page 8).¹ After stating that the '251 patent "discloses the benefits of combining viral antigens and cancer antigens with a stress protein, and Young discloses that those antigens in fusion with one another provide good T cell reactivity upon immunization," the Examiner argues that one "would have been motivated to create a fusion protein comprising the influenza or cancer antigen in fusion with the stress protein so that the two moieties would be sure to stay together throughout the antigen presentation process, ensuring a better response" (Office Action at page 8).

To establish a *prima facie* case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Third, the prior art reference(s) must teach or suggest all the claim limitations. MPEP at 2143. Neither the first nor the second requirements are met with respect to claim 54, which now carries the limitation previously recited in claim 56, or claims 57, 58, or 60.

Nothing in Young or the '251 patent provides the motivation to modify or combine their teachings, and the Examiner appears to clearly recognize that. Instead of using the references to establish the requisite motivation, the Examiner argues, as noted above, that one of ordinary skill in the art would have been motivated to make the claimed fusion proteins because the two moieties within a fusion protein "would be sure to stay together throughout the antigen presentation process" and this would ensure "a better response" (Office Action at page 8). Thus,

¹ The complexes disclosed in the '251 patent are strictly limited to non-covalent complexes; fusion proteins are not suggested in any way.

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the Examiner's case of *prima facie* obviousness relies on the "skill in the art" component. This component is rarely sufficient to support an obviousness rejection, and it cannot do so here.

In *Al-Site Corp. v. VSI Intern., Inc.*, VSI attempted to invalidate Al-Site's patent by arguing, *inter alia*, that the claimed subject matter (a hanger for displaying non-prescription eyeglasses) was obvious. 174 F.3d 1308 (Fed. Cir. 1999). VSI was unable to point to any specific teaching or suggestion for making the claimed combination of elements, so it relied instead on what it presumed to be the level of ordinary skill in the art at the time of the invention to supply the missing suggestion to combine. VSI's argument failed, the court stating (*Al-Site* at 1324):

In the first place, the level of skill in the art is a prism or lens through which a judge or jury views the prior art and the claimed invention. This reference point prevents these deciders from using their own insight or, worse yet, hindsight, to gauge obviousness. Rarely, however, will the skill in the art component operate to supply missing knowledge or prior art to reach an obviousness judgment. See *W.L. Gore & Assocs., Inc. v. Garlock, Inc.*, 721 F.2d 1540, 1553, 220 USPQ 303, 312-13 (Fed. Cir. 1983) ("To imbue one of ordinary skill in the art with knowledge of the invention in suit, when no prior art reference or references of record convey or suggest that knowledge, is to fall victim to the insidious effect of a hindsight syndrome wherein that which only the inventor taught is used against its teacher."). Skill in the art does not act as a bridge over gaps in substantive presentation of an obviousness case, but instead supplies the primary guarantee of objectivity in the process.

The Examiner is attempting to bridge an important gap in this case with nothing more than an unsupported statement concerning what one of ordinary skill in the art might think. But, as in *Al-Site*, the gap cannot be bridged (VSI supported their contention with prior art patents and still failed). The facts cannot support the conclusion that one of ordinary skill in the art would have been motivated to use the influenza antigens disclosed in the '251 patent as part of a fusion protein. There is simply nothing upon which to base the presumption that the components of a fusion protein would "stay together" any better than the components of the protein complexes in the '251 patent. Indeed, the '251 patent teaches that non-covalent hsp-containing complexes occur naturally in patients. For example, the '251 patent teaches (8:57-67; emphasis added):

Should not
be
indented

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The methods of the invention comprise methods of eliciting an immune response in an individual in whom the treatment or prevention of infectious diseases or cancer is desired by administering a composition comprising an effective amount of a complex, in which the complex consists essentially of a hsp noncovalently bound to an antigenic molecule. In a preferred embodiment, the complex is autologous to the individual; that is, the complex is isolated from either [from] the infected cells or the cancer cells for precancerous cells of the individual himself (e.g., preferably prepared from infected tissues or tumor biopsies of the patient).

The complexes can also be isolated from healthy individuals. The '251 patent teaches that (6:12-14; emphasis added):

Alternatively, the hsp and or the antigenic molecule can be isolated from ... others [*i.e.*, one other than the patient]

Thus, the non-covalent protein complexes described in the '251 patent are stable under normal physiological conditions (otherwise they could not be isolated from healthy individuals), as well as under extreme physiological conditions (*i.e.*, when the cell is infected, cancerous, or exposed to another form of stress, such as heat; that is, after all, how hsps exert their protective effect – by complexing with other proteins). There is no evidence that fusion proteins would be more stable (a covalent bond is not invincible), nor is there any evidence that those of ordinary skill in the art would presume as much. This is the Examiner's supposition, made with full knowledge of Applicants' success with fusion proteins, but it cannot be fairly attributed to one of ordinary skill in the art at the time the invention was made.

Furthermore, even if there was reason to presume that fusion proteins will "stay together," nothing suggests that they will produce a "better response" than the non-covalent complexes of the '251 patent. To the contrary, it was known in the art that recombinant proteins (which fusion proteins would necessarily be) are not as immunogenic as polymeric proteins (such as non-covalently bound protein complexes). For example, in the background section of his U.S. Patent (No. 4,918,166, a copy of which is attached as Exhibit C), Kingsman states (emphasis added):

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A substantial disadvantage of most antigens produced by recombinant DNA techniques for vaccines is that they are usually made as simple monomeric proteins. This is not the ideal configuration for an immunising antigen as it does not readily permit the cross-linking of the components of the immune system that is required for maximum stimulation of humoral and cellular immunity. An ideal immunogen is a polymer of multiple antigenic determinants assembled into a high molecular weight carrier. A good immunogen should also have the maximum number [of] epitopes exposed. This is best achieved by presenting multiple copies of the antigen on the surface of a particle.

In view of the foregoing, it should be clear that there is no motivation to combine Young and the '251 patent to arrive at the subject matter now claimed. On this basis alone, the rejection for obviousness should be withdrawn.

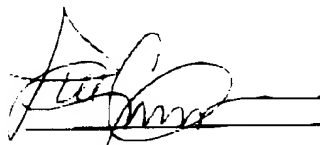
The prior art of record also fails to supply a reasonable expectation of success. The consistent criterion for determining obviousness has been whether the prior art suggests to one of ordinary skill in the art that the claimed invention should be carried out and would have a reasonable likelihood of success – both the suggestion and the expectation of success must be found in the prior art. *In re Dow Chemical Co.*, 837 F.2d 469 (Fed. Cir. 1988). As one of ordinary skill is charged with knowledge of the entire body of technological literature, one would necessarily be aware of the teaching of Kingsman (see the excerpt above). Given that teaching, Applicants had, at most, only an invitation to experiment, and that is not sufficient to maintain a rejection for obviousness. The prior art cannot provide a reasonable expectation for success with fusion proteins containing the particular antigens now claimed. This ground for rejection should be withdrawn.

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Attached is a marked-up version of the changes being made by the current amendment. Applicants ask that all claims be examined. Enclosed is a check for \$445 for the Petition for Extension of Time fee. Please apply any other charges, or any credits, to Deposit Account No. 06-1050.

Respectfully submitted,

Date: June 11 2001

Lee Crews, Ph.D.
Reg. No. 43,567

Fish & Richardson P.C.
225 Franklin Street
Boston, MA 02110-2804
Telephone: (617) 542-5070
Facsimile: (617) 542-8906

20260561.doc

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Version with markings to show changes made

In the claims:

Claims 55, 56, and 60 have been cancelled.

Claims 54, 57-59, and 61-67 have been amended as follows.

54. (Amended) A fusion protein comprising an antigen of an influenza virus, or an antigenic portion thereof, and a stress protein, or a portion [of the amino acid sequence of the stress protein] thereof, wherein the [stress] fusion protein [or the portion thereof is able to induce a cell mediated cytolytic] induces an immune response against the antigen in a mammal to whom the fusion protein is administered.

57. (Amended) The fusion protein of claim [56] 54, wherein the antigen of the influenza virus is [selected from the group consisting of] hemagglutinin, nucleoprotein, neuraminidase, M1, M2, PB1, PB2, or PA [and a combination thereof].

58. (Amended) The fusion protein of claim 54, wherein the fusion protein is [selected from the group consisting of] encoded by plasmid pET65MP/NP-B [and] or plasmid pET65MP/NP-D.

59. (Amended) The fusion protein of claim 54, wherein the antigen includes a [cytolytic T cell] CTL epitope.

61. (Amended) [The] A fusion protein [of claim 54 wherein the stress protein is] comprising an antigen of the influenza virus, or an antigenic portion thereof, and a bacterial stress protein, or a portion thereof, wherein the fusion protein induces an immune response against the antigen in a mammal to whom the fusion protein is administered.

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62. (Amended) The fusion protein of claim 61, wherein the bacterial stress protein is a mycobacterial stress protein.

63. (Amended) [The] A composition comprising the fusion protein of [any one of claims 54-62, in combination with] of claim 54 and a pharmaceutically acceptable excipient, carrier, diluent, or vehicle.

64. (Amended) A method of inducing [a cell mediated cytolytic] an immune response against an antigen of an influenza virus, the method comprising administering the fusion protein of claim 54 to a vertebrate in an amount effective to induce an immune response [a fusion protein according to claim 54] against the antigen.

65. (Amended) The method of claim 64, wherein the fusion protein is administered in combination with a pharmaceutically acceptable excipient, carrier, diluent, or vehicle.

66. (Amended) [The] A method of inducing an immune response against an antigen of the influenza virus, the method comprising administering the fusion protein of [claim 64 with the fusion protein of any one of claims 55-62] claim 58 to a vertebrate in an amount effective to induce an immune response against the antigen.

67. (Amended) The method of claim 66, wherein the fusion protein is administered in combination with a pharmaceutically acceptable excipient, carrier, diluent, or vehicle.

The Neuraminidase

The neuraminidase, NA, is a tetramer of identical subunits. It has a molecular weight of approximately 240,000, and it appears in the electron microscope to have a box-shaped head on a thin stalk that is anchored in the viral membrane. Both head and stalk are N-glycosylated at two to five sites, depending on the particular NA. The molecule projects from the viral surface by about 120 Å; it is therefore similar in length to the 135-Å HA. NA is anchored in the membrane by an NH₂-terminal hydrophobic region that also serves as its signal sequence. Tetrameric heads, enzymically and antigenically indistinguishable from the intact molecule, can be released from the stalks by proteolytic digestion, and their three-dimensional structures have been determined by x-ray crystallography (32). These analyses show that each subunit consists of six four-stranded, W-shaped β -sheets in which the strands are connected by reverse turns. The fourth strand of each sheet is connected across the upper face to the first strand of the following sheet. The first strands of the sheets nearest the center of the subunit are almost parallel with the fourfold axis of symmetry of the complete tetramer, and the sheets gradually twist so that the fourth strands are almost at right angles to the first. This arrangement gives the subunit the shape of a six-bladed propeller (Fig. 29).

NA catalyzes the cleavage of α -(2,3) and α -(2,6) glycosidic linkages between terminal sialic acids and the penultimate saccharide residues of glycoprotein and glycolipid carbohydrate side chains (33). As a result, it removes the sialic acid residues recognized by HA and destroys the influenza receptor activity of glycoconjugates. During viral replication, NA activity ensures efficient release of newly assembled viruses from the desialylated infected cell surface, and the production of desialylated viral glycoproteins prevents aggregation of virus particles by inappropriate HA/receptor interactions. These essential roles make NA a target in antiviral drug design programs based on the structure of the enzyme active site and its interactions with sialic acid.

X-ray studies of crystalline NA/sialic acid complexes show that the enzymatic active site is structurally quite different from the sialic acid receptor recognition site in the HA (157). The active site is located on the membrane distal surface near the axis relating the six β -sheets. It contains seven charged residues, which are completely conserved in all influenza NAs. The carboxyl group of the α -anomeric sialic acid reaction product interacts in an equatorial orientation with three conserved arginine residues, and the 5-*N*-acetyl and 6-glyceryl groups also interact with conserved amino acid side chains. Detailed examination of the site and computer predictions of modifications to bound sialic acid that might increase interactions with the enzyme showed that the 4-hydroxyl group, which is directed toward conserved glutamic acid 119, was a candidate for modification. Introduction of a

guanidynyl group at this position in a previously characterized inhibitory sialic acid analog has led to the production of a potent and specific neuraminidase inhibitor and anti-influenza compound (157).

Antigenic Variation

Analyses of the structures of NA and HA have been directed in large measure toward understanding the molecular basis of antigenic variation—a notorious property of influenza virus. As surface glycoproteins, HA and NA are exposed to interactions with antibodies that block infectivity. During an influenza pandemic period they both vary considerably, and to about the same extent. The regions of the molecules subject to variation can be identified by locating the positions of changes in amino acid sequence on the three-dimensional structures of HA and NA. Such studies indicate that the residues primarily involved in both glycoproteins are components of their membrane distal surfaces. The antigenic significance of the changes can be inferred from similar analyses of the sequence changes in antigenic variant glycoproteins, selected by growing virus in the presence of monoclonal antibodies that block infectivity. There is an excellent correspondence between the locations of changes in natural variants and those selected *in vitro* (163). X-ray studies of monoclonal antibody selected variants of both HA and NA glycoproteins have also shown that the site of the amino acid substitution in the variant defines the site of antibody binding. That is, the structural differences between wild type and variant HAs and NAs are confined to the location of the substituted amino acid. The most direct formal proof of this conclusion has come from x-ray studies of NA-monoclonal Fab complexes (31). They indicate that about 700 Å² of the membrane-distal NA surface are buried in the bound antibody fragment, including approximately 17 amino acid residues in five discontinuous loops. All the amino acids substituted in different variant NAs selected by the antibody are components of the buried surface, directly proving the conclusion that such substitutions define the location of the epitope. Structural changes detected in the bound NA, although small, may be important for the strength of the antibody/antigen interaction.

The Ion Channel Protein, M2

The influenza A virus ion channel, a relatively minor component of the viral membrane, is formed by the nonglycosylated transmembrane polypeptide, M₂ (79). This protein is the smaller product of the gene that encodes the matrix protein, M₁. The channel is a tetramer that contains two noncovalently associated dimers, each with an internal disulfide link (83,148). The polypeptide chain is oriented so that, of the 97 amino acids, the NH₂-terminal

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13,588 nucleotides. In this chapter no attempt has been made to cite the original publication of the several hundred influenza virus nucleotide sequences obtained and deposited in the EMBL/GenBank database. Although the nucleotide sequence of the vRNA segments provides the definitive proof of the coding assignment of the genes, these assignments were made earlier by ingenious experiments using genetic methods and hybrid-arrest of translation strategies [reviewed in (211)].

The first 12 nucleotides at the 3' end and the first 13 nucleotides at the 5' end of each vRNA segment are conserved in all eight RNA segments (see Fig. 15). Recent studies using RNAs of altered sequence and reconstitution of the transcriptase activity indicate a promoter role for these sequences in transcription (see below).

The Polymerase Proteins and their Genes

The three largest RNA segments encode the PB1, PB2, and PA proteins (apparent M_r ~96,000, 87,000, and 85,000, respectively) (158,206). Because of anomalous migration of influenza virus RNA segments on gels, the proteins encoded by RNA segments 1 and 3 from different subtypes are different. Therefore, the proteins are named after their behavior on isoelectric focusing gels: two P proteins were found to be basic (PB1 and PB2) and one acidic (PA) (144, 372,411). RNA segments 1 and 2 are both 2,341 nucleotides in length and code for proteins of 759 amino acids (PB2) and 757 amino acids (PB1), respectively, and PB1 and PB2 are basic proteins at pH 6.5 with a net charge of +28. As the sizes of the two polypeptides PB1 and PB2 are very similar, the ability to separate them on polyacrylamide gel electrophoresis must be due to factors such as differential binding of SDS. RNA segment 3 is 2,233 nucleotides in length and codes for a protein of 716 amino acids that has a charge of -13.5 at pH 6.5. Of the three polymerase proteins, PB1 has been implicated in catalytic activity including nucleotide polymerization and chain elongation (35). All viral RNA-dependent RNA polymerases examined to date contain four conserved motifs (301), and a mutational analysis of these conserved motifs in PB1 indicates that invariant residues in each of these motifs are critical in PB1 function (25). The three P proteins form a complex in the cytoplasm and nucleus of cells that is largely resistant to disruption by normal immunoprecipitation buffers and the complex sediments on sucrose gradients at 11 to 22S (76). After synthesis in the cytoplasm, the P proteins are transported to the nucleus, possibly as a complex. However, expression of the individual P proteins from cDNA has shown that each P protein migrates to the nucleus and thus contains a karyophilic signal (4,163,354). A fuller description of the known functions of the PB2, PB1, and PA proteins in RNA synthesis is presented below.

Influenza B and C viruses also encode three proteins which show extensive sequence identity and homology to those of influenza A virus, and it is presumed these proteins have roles and properties similar to those of influenza A virus.

The Nucleocapsid Protein and its Gene

The NP is the major structural protein that interacts with the RNA segments to form the RNP. Nucleocapsid protein is also one of the type-specific antigens that distinguishes between the influenza A, B, and C viruses. The NP protein is also the major target of cross-reactive cytotoxic T lymphocytes generated against all influenza virus subtypes in mice and man [reviewed in (418)]. The NP protein is encoded by RNA segment 5 which for influenza A virus is 1,565 nucleotides in length. The NP protein contains 498 amino acids with a predicted M_r of 56,101; the protein is rich in arginine residues and has a net positive charge of +14 at pH 6.5 (410). However, unlike what might be expected for a protein that interacts with the acidic phosphate residues of RNA, there are no clusters of basic residues which suggest that probably many regions of the NP molecule interact with RNA. The NP is phosphorylated (293, 306,308), but it is not clear what percentage of NP molecules are phosphorylated or whether phosphorylation is essential for function. After synthesis in the cytoplasm, NP molecules are transported to the nucleus. Nuclear targeting of NP is an intrinsic property of the protein (235,332), and a molecular analysis has indicated that NP residues 327 to 345 are sufficient and necessary to target NP to the nucleus (74). Interestingly, the NP karyophilic sequence has little resemblance to the prototype basic residue sequence identified in SV40 T-antigen (165,166).

The mechanism of assembly of the nucleocapsid is poorly understood. Both virion RNA (- strand) and template (+ strand) RNAs are found associated with NP molecules, whereas the viral mRNAs (+ strand) are not encapsidated (121,302); therefore, there must exist a mechanism that prevents NP from association with mRNAs. Although NP protein synthesized from cDNA in bacteria associates with many RNA species (and DNA), this interaction lacks specificity (184). Influenza virus must form RNPs with all eight RNA segments, and if there is a common nucleation site it may lie in the conserved 3' and 5'-terminal nucleotides which are found on vRNA and template RNA strands. Because template (+ strand) RNAs are encapsidated with NP, whereas mRNAs are not, it seems possible that the putative nucleation site includes the 13 common 5' vRNA nucleotides whose complement is lacking in mRNAs (see below).

The influenza B virus NP protein contains 560 amino acids and is 47% homologous to influenza A virus NP, and the influenza C virus NP protein contains 565 amino acids

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nent of virions and has ion channel activity. Three mRNA transcripts have been identified that are derived from influenza A virus RNA segment 7: a colinear transcript encoding M_1 protein; a spliced mRNA encoding the M_2 protein; and an alternatively spliced mRNA ($mRNA_3$) which has the potential to encode a 9 amino acid peptide, but it has not been recognized. Influenza B virus RNA segment 7 encodes two polypeptides using tandem cistrons, the matrix protein M_1 , and the 109-residue BM_2 protein of unknown function. The equivalent RNA segment in influenza C virus (RNA segment 6) encodes the matrix protein which the available evidence indicates is translated from a spliced mRNA.

RNA Segment 7 Gene Structure and Encoded Proteins**Influenza A Virus**

RNA segment 7 of influenza A virus encodes two known proteins, M_1 and M_2 . The gene is 1,027 nucleotides in chain length and has one large ORF of 237 residues which encodes the M_1 protein (6,215,409) (Fig. 8). A colinear transcript mRNA encodes the M_1 protein, whereas the M_2 protein is encoded by a spliced mRNA (157,218). The M_2 mRNA contains a 51-nucleotide virus-specific leader sequence, a 689-nucleotide intron, and a 271-nucleotide [excluding poly(A) tail] body region. The leader sequence of the M_2 mRNA body region encodes 88 residues in the +1 reading frame and overlaps the M_1 protein by 14 residues. A second alternatively spliced mRNA ($mRNA_3$) has also been identified and it has a 5' leader sequence of 11 virus-

specific nucleotides, and shares the same 3' splice site as the M_2 mRNA. No evidence has been obtained to indicate that M_3 mRNA is translated but if it is it would yield a 9-residue peptide identical to the C-terminus of the M_1 protein (218).

The M_1 protein contains 252 residues ($M_r=27,801$) and requires 0.5 M KCl to be solubilized (219), although it is not an integral membrane protein. The M_1 protein is a type-specific antigen of influenza virus and comparison of its predicted amino acid sequence among influenza A virus subtypes indicates it is highly conserved [reviewed in (207)].

Influenza B Virus

RNA segment 7 of influenza B virus encodes two known proteins, M_1 and BM_2 . The gene is 1,191 nucleotides in length and contains two ORFs (Fig. 9). The first ORF in the 0 reading frame begins at the AUG codon at nucleotides 25 to 27 and continues to a termination codon at nucleotides 769 to 771. This ORF encodes the 248 amino acid M_1 protein, 63 residues of which are identical with those of the influenza A virus M_1 protein (41). A second ORF in the +2 frame, overlapping the M_1 protein ORF by 86 residues, has a coding capacity of 195 residues and is designated BM_2 (41,135,148). A polypeptide, BM_2 , derived from the BM_2 ORF was identified in cells infected with influenza B virus by using an antisera generated to a β -galactosidase- BM_2 ORF fusion protein (148). The BM_2 protein appears to be a soluble and cytoplasmically located protein of unknown function. In an attempt to understand the mechanism by which the BM_2 protein is synthesized, a mutational analysis of the cloned RNA segment 7 was performed. The data indicate that the BM_2 protein initiation codon overlaps with the termination codon of the M_1 protein in a translational stop-start pentanucleotide UAAUG, and that expression of the BM_2 protein requires termination of M_1 synthesis adjacent to the 5' end of the BM_2 coding region: thus, termination of translation and the reinitiation event are tightly coupled (148) (see Fig. 9). Reinitiation of translation at downstream AUG codons in eukaryotes is uncommon but has been found to occur with artificially constructed bicistronic mRNAs (191). However, in prokaryotes, coupled tandem cistrons with the termination codon of one gene overlapping the initiation codon for a downstream gene is a common situation for coordinating regulated bacterial genes (e.g., *trp* operon) [reviewed in (278)].

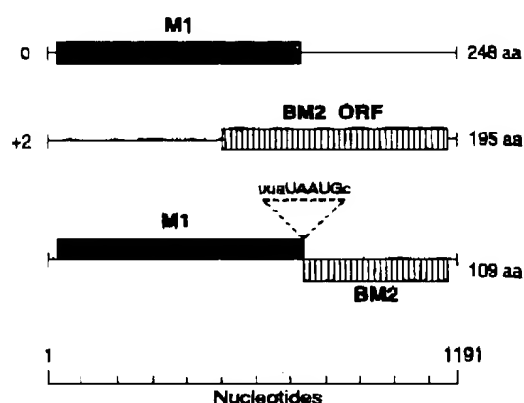


FIG. 9. Schematic representation of the ORFs in influenza B virus RNA segment 7. **Top two lines:** the ORF encoding the M_1 protein contains 248 residues, and the BM_2 ORF consists of 195 residues (41). **Third line:** the extent of the BM_2 ORF used to translate the BM_2 protein found in influenza B virus infected cells. The pentanucleotide at which M_1 translation stops and M_2 translation starts is shown in capital letters. From Horvath et al. (148), with permission.

Influenza C Virus

RNA segment 6 of influenza C virus contains 1,180 nucleotides and contains a single ORF of 374 residues that could encode a polypeptide of $M_r \sim 41,700$ (415) (Fig. 10). However, the M_1 protein of influenza C virus has an $M_r \sim 28,000$ on polyacrylamide gels (64,362). Hybrid-selec-

EXHIBIT B

Attorney's Docket No.: 12071-011002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Lee Mizzen *et al.*
Serial No. : 08/977,787
Filed : November 25, 1997
Title : IMMUNE RESPONSES USING COMPOSITIONS CONTAINING STRESS
PROTEINS

Art Unit : 1643
Examiner : Mary K. Zeman

Commissioner for Patents
Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. § 1.131

We, Lee Mizzen, Lawrence S.D. Anthony, Huacheng Bill Wu and Marvin Siegel, do hereby declare that:

1. We are co-inventors of the subject matter described in the above-identified patent application ("subject application").
2. All of the work described within this Declaration was performed by ourselves or on our behalf and under our direction.
3. We have reviewed our laboratory records, including the Exhibit submitted herewith, and readily conclude that compositions of matter and methods, as claimed in the subject application, were conceived prior to January 1, 1996. Further, due diligence was exercised from this time period until the invention was actually reduced to practice.
4. Prior to January 1, 1996, we conceived of a fusion protein containing an antigen and a stress protein to induce a cell mediated cytolytic immune response against the antigen. Experiments by us confirmed that such a fusion protein could be prepared and used for *in vivo* immunization.
5. The following Exhibit (annexed hereto) represents laboratory notebook pages and material requisition forms kept in the regular course of business at StressGen Biotechnologies

CERTIFICATE OF MAILING BY FIRST CLASS MAIL

I hereby certify under 37 CFR §1.8(a) that this correspondence is being deposited with the United States Postal Service as first class mail with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, Washington, D.C. 20231.

Date of Deposit

Signature

Typed or Printed Name of Person Signing Certificate

Applicant : Lee Mizzen *et al.*
Serial No. : 08/977,787
Filed : November 25, 1997
Page : 2

Attorney's Docket No.: 12071-011002

Corp. The dates have been removed from the copies submitted herewith. It is our understanding, based on discussions with assignee's representatives, that this is a permissible Patent Office practice. The Exhibit discloses the initiation of preparation of a fusion protein as described above.

6. The work described in the Exhibit was conducted in the United States of America or Canada.

7. In summary, upon review of our laboratory records, of which the enclosed pages are representative, we have concluded that, at least prior to January 1, 1996, we had conceived of the compositions of matter and methods as described and claimed within the subject application. Further, our conception of the invention led to further research, diligently undertaken, resulting in an actual reduction to practice.

We further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that the making of willfully false statements and the like is punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and may jeopardize the validity of any patent issuing from this patent application.

23 May 2001
Date

Lee Mizzen
Lee Mizzen

23 May 2001
Date

Lawrence S.D. Anthony
Lawrence S.D. Anthony

23 May 2001
Date

Huacheng Bill Wu
Huacheng Bill Wu

23 May 2001
Date

Marvin Siegel
Marvin Siegel

EXHIBIT C

United States Patent [19]

Kingsman et al.

[11] Patent Number: 4,918,166
 [45] Date of Patent: Apr. 17, 1990

[54] PARTICULATE HYBRID HIV ANTIGENS

[75] Inventors: Alan J. Kingsman; Susan M. Kingsman, both of Islip; Sally E. Adams, Kidlington, all of United Kingdom

[73] Assignee: Oxford Gene Systems Limited, Oxford, England

[21] Appl. No.: 112,083

[22] Filed: Oct. 26, 1987

Related U.S. Application Data

[63] Continuation of Ser. No. 36,888, Apr. 10, 1987.

[51] Int. Cl. C07K 15/04; C07K 15/14; C07K 17/00; G01N 33/569

[52] U.S. Cl. 530/350; 424/89; 530/395; 530/403; 530/806; 530/812; 530/824; 530/826; 435/5

[58] Field of Search 435/5, 172.3, 320; 530/350, 387, 395, 412, 403, 806, 812, 824, 826; 424/89

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Primary Examiner—Christine M. Nucker
 Attorney, Agent, or Firm—Allegretti & Witcoff, Ltd.

ABSTRACT

[57] Fusion proteins comprise a 77 first amino acid sequence and a second amino acid sequence. The first amino acid sequence is derived from a retrotransposon or an RNA retrovirus and confers on the fusion protein the ability to assemble into particles; an example is the product of the TYA gene of the yeast retrotransposon Ty. The second amino acid sequence is an HIV antigen. So particles formed of the fusion proteins may be useful in vaccines or in diagnostic or purification applications.

3 Claims, 13 Drawing Sheets

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PARTICULATE HYBRID HIV ANTIGENS

This is a continuation-in-part of Ser. No. 07/036,888 filed Apr. 10, 1987.

FIELD OF INVENTION

The present invention relates to particulate Human Immunodeficiency Virus (HIV) antigens. In particular it relates to hybrid particles composed of fusion proteins comprising amino acid sequences encoded by the TYA gene of the yeast retrotransposon Ty and amino acid sequences encoded by HIV, vectors containing the genes for the fusion proteins, vectors for the high level expression of the hybrid particles and a method for the production of these particles in yeast. The Ty:HIV hybrid particles may be used as an HIV vaccine or as components of HIV diagnostic tests.

BACKGROUND ART

HIV, also known as LAV or HTLV III, is the causative agent of Acquired Immuno-Deficiency Syndrome (AIDS) (Barre-Sinoussi et al. 1983 Science 220, 868; Gallo et al. 1984 Science 224, 500; Levy et al. 1984 Science 225, 840; Clavel et al. 1986 Nature 324 691). At present there is no cure for AIDS nor is there a vaccine available. The genetic organization and the entire nucleotide sequence of HIV is known (Ratner et al. 1985 Nature 313, 277; Wain-Hobson et al. 1985 Cell 40, 9; Muesing et al. 1985 Nature 313 450; Sandoz-Pescador et al. 1985 Science 227 484). HIV is a lentivirus-like retrovirus with gag, pol and env genes like other retroviruses but it also contains additional coding sequences, sor, tat, art/trs and 3' orf that are involved in various aspects of virus replication and expression (Sodroski et al. 1985 Science 227, 171; Sodroski et al. 1986 Nature 319, 555; Sodroski et al. 1986 Nature 321, 412; Feinberg et al. 1986 Cell 44, 807) although the functions of sor and 3' orf are unclear.

Three general approaches can be used to produce an HIV vaccine. First, large amounts of HIV can be grown and inactivated to provide antigen. Second, recombinant DNA techniques can be used to produce HIV antigens either as simple monomeric proteins (e.g. Putney et al. (1986) Science 234 1392; Laskey et al. 1986 Science 233 209) or as vaccinia virus hybrids, although it is not clear that general use of a live vaccinia based system will ever be considered safe (e.g. Chakrabarti et al. 1986 Nature 320, 535; Zagury et al. 1987 Nature 326, 249). Third, synthetic peptides might be useful (Kennedy et al. 1986 Science 231 1556; Chan et al. EMBO J. 5 3065).

Most of the work to date on producing HIV antigens has focussed on the production of the two surface glycoproteins encoded by the env gene, gp120 and gp41 (Putney et al. op. cit.; Laskey et al. op. cit.; Cera et al. 1986 EMBO J. 5 3051) although there has been some work on other antigens e.g. tat III (Aldovini et al. 1986 PNAS 83 6672), sor (Kao et al. 1986 Science 231 1553), pol (Veronese et al. 1986 Science 233 1289; Kramer et al. 1986 Science 231 1580). The production of HIV antigens for vaccines or diagnostics and research material by recombinant DNA technology has three key advantages over production based on propagation of the virus. First, it is safe. Second, high yields can be achieved (Putney et al. op. cit.) by using high efficiency expression systems. Third, it is versatile in that antigenic domains that might normally be concealed may be ex-

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posed, a vaccine antigen could be marked with some other antigen to distinguish vaccination from infection or composite antigens might be produced.

A substantial disadvantage of most antigens produced by recombinant DNA techniques for vaccines is that they are usually made as simple monomeric proteins. This is not the ideal configuration for an immunising antigen as it does not readily permit the cross-linking of the components of the immune system that is required for maximum stimulation of humoral and cellular immunity. An ideal immunogen is a polymer of multiple antigenic determinants assembled into a high molecular weight carrier. A good immunogen should also have the maximum number epitopes exposed. This is best achieved by presenting multiple copies of the antigen on the surface of a particle. For this reason it would be desirable to develop polyvalent, particulate carrier systems for immunising antigens.

DISCLOSURE OF THE INVENTION

An entirely novel polyvalent antigen carrier particle, based on the ability of the pI protein encoded by the TYA gene of the yeast retrotransposon Ty to form 60 nm particles known as Ty-VLPs (virus like particles), is the subject of U.K. Patent Application No. 8626148 [having a common assignee with this application.] pI fusion proteins can be produced by construction of appropriate TYA hybrid genes comprising some of the coding region of TYA and the coding region of any antigen. These fusion proteins form hybrid Ty-VLPs and present the added antigen in a high molecular weight polyvalent particulate form that is ideal for the stimulation of the mammalian immune response. In the present invention this technology is applied to the production of particulate HIV antigens.

According to a first aspect, the present invention provides a fusion protein capable of assembling into a particle, the fusion protein comprising a first amino acid sequence and a second amino acid sequence, wherein the first amino acid sequence is substantially homologous with a particle-forming protein encoded by a retrotransposon or an RNA retrovirus and wherein the second amino acid sequence is substantially homologous with an HIV antigen, and wherein the second amino acid sequence does not form an amino acid sequence naturally directly fused to the first amino acid sequence by the said retrotransposon or RNA retrovirus.

According to a second aspect, the invention provides a particle comprising a plurality of fusion proteins, each fusion protein comprising a first amino acid sequence and a second amino acid sequence, wherein the first amino acid sequence is substantially homologous with a particle-forming protein encoded by a retrotransposon or an RNA retrovirus and wherein the second amino acid sequence is substantially homologous to an HIV antigen and not naturally fused to the first amino acid sequence by the said retrotransposon or RNA retrovirus.

These particles may be referred to as particulate HIV antigens.

Such particles will generally be substantially pure, by which is meant at least 5%, 10%, 20%, 50%, 80%, 90%, 95% or 99% by weight pure, in increasing order of preference.

A given particle may be composed of a plurality of different fusion proteins; that is to say fusion proteins having different second amino acid sequences from

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each other, thereby presenting different HIV antigens. Two, three or even more different second amino acid sequences may be present in a particle.

The first amino acid sequence may be the product of the yeast Ty TYA gene, the product of copia and copia-like elements from insects or the gag gene of a RNA retrovirus.

Retroviruses includes Human Immunodeficiency Virus I and II (HIV-I, HIV-II), Human T-cell Lymphotropic Virus I and II (HTLV-I, HTLV-II), Murine Leukaemia Virus, Moloney Murine Leukaemia Virus, Mouse Mammary Tumour Virus, Avian Leukosis Virus, SiV, Feline Leukaemia Virus, Human B-cell Lymphotropic Virus, and Bovine Leukaemia Virus. Retrotransposons indicated above, include the Ty element of yeast, the copia and copia-like elements of insects such as *Drosophila melanogaster*, VL30 in mice and IAP genes in mice.

Preferred retrotransposons include the yeast retrotransposon Ty. It has previously been shown that Ty directs the synthesis of 60 nm virus-like particles (Ty-VLPs) (Mellor et al. 1985a Nature 318,513). It has now been discovered, among other things, that the p1 protein, encoded by the TYA gene does not appear to require further processing to produce Ty-VLPs. Therefore the Ty-encoded amino acid sequence is preferably the p1 protein encoded by the TYA gene. It is known (Fulton et al. NAR 13(11) 1985 4097) that both classes (I and II) of Ty make p1; so either class may be used.

The Ty-encoded amino acid sequence need not be the whole of the p1 protein; instead it may be a part of the p1 protein encoded by a part of the TYA gene, which part is capable of directing the synthesis of Ty virus-like particles (Ty-VLPs). Preferably the Ty-encoded amino acid sequence is derivable from the class I Ty element known as Ty1-15. The stop codon at the end of the TYA gene is preferably not included; if it is included, however, fusion protein may continue to be expressed, albeit at low yield, as it appears that the stop codon may be ignored with a frequency of about 1 in 20 times by the frameshifting mechanism described by Wilson et al. (NAR 14(17) 1986 7001).

The present invention is thus at least in part based on the discovery that Ty protein p1, the product of the TYA gene (Dobson et al. 1984 EMBO J 3, 1115) is sufficient to produce Ty-VLPs and that p1, among other things, is capable of being used in the formation of particles composed of fusion proteins.

The second amino acid sequence may be substantially homologous with (which term clearly includes "identical to") any HIV antigen. The HIV antigen may be an HIV-I or HIV-II antigen. It may be glycosylated or otherwise modified, whether by a natural post-transcriptional modification mechanism or otherwise (e.g. by chemical synthesis). In particular, the second amino acid sequence may be an HIV surface glycoprotein. Such glycoproteins (or at least some of them) are believed to be encoded by the env gene. Instances of these glycoproteins include gp120 and gp41.

Thus this invention includes as the second protein HIV antigens having substantially the same antigenicity as HIV antigen p24, p41, or p120.

A first part of the second amino acid sequence may be a linker sequence, which may in some circumstances be readily cleavable. The remainder of the second amino acid sequence may thus be cleaved off in a purification step.

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Particulate antigens in accordance with the invention may therefore be useful in the preparation of vaccines, which form a further aspect of the invention. The vaccine may comprise a particulate antigen and a physiologically acceptable non-toxic carrier, such as sterile physiological saline or sterile PBS. Sterility will generally be essential for parenterally administrable vaccines. One or more appropriate adjuvants may also be present. Examples of suitable adjuvants include muramyl dipeptide, aluminum hydroxide and saponin.

It should be noted that vaccines in accordance with the invention may present more than one antigen. Either a cocktail of different particulate antigens may be used, or a homogeneous population of particulate antigens having more than one epitope could be used (prepared, for example, by allowing a mixture of different hybrid proteins to aggregate into particles or by expressing more than one particulate antigen in the same cell); alternatively, a vaccine could contain a mixture of these sorts of particulate antigens.

In a further aspect, the invention provides nucleic acid comprising a first nucleotide sequence and second nucleotide sequence, wherein the first nucleotide sequence is substantially homologous with or complementary to genetic material in a retrotransposon or RNA retrovirus encoding a particle-forming protein, and wherein the second nucleotide sequence which encodes, as HIV antigen, to form a fusion protein which is not naturally produced by the said retrotransposon or RNA retrovirus.

It will generally be the case that the nucleic acid will be capable of being expressed without splicing or antitermination events. There will generally be no frameshifting.

In certain embodiments of the invention, we provide a TYA gene derivative that can be fused to an HIV antigen coding sequence to produce a TYA fusion gene. The TYA fusion gene produces a fusion protein that assembles into hybrid Ty-VLPs. These hybrid Ty-VLPs constitute a high molecular weight particulate antigen presentation system that can be produced in very high yields and that can be purified by simple physical procedures.

Further according to the present invention we provide an expression vector including nucleic acid as defined above. An example is pMA5620, which includes TYA gene derivative, and which directs the high level production of hybrid Ty-VLPs in yeast.

Expression vectors in accordance with the invention will usually contain a promoter. PGK is a preferred promoter, but any other promoter may be used if necessary or desirable. Examples includes GAPD, GAL1-10, PHOS, ADHI, CYC1, Ty delta sequence PYK and hybrid promoters made from components from more than one promoter (such as those listed).

The invention also includes host cells for example bacterial cells such as *E. coli*, yeast cells such as *S. cerevisiae*, or animal cells such as COS or CHO cells containing appropriate expression vectors.

Because of the polyvalent nature of the particulate antigens it is likely that it will be easier to produce antibodies than with conventional antigens and that these antibodies will have specific characteristics. The invention thus further provides antibodies raised against particulate antigens of the invention. The antibodies may be polyclonal (obtained for example by injecting antigens into a rabbit) or monoclonal antibodies produced by hybridoma cells in accordance with the inven-

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tion. Because of the polyvalent nature of the particulate antigens it is likely that in vitro immunisation can be achieved more readily than with other forms of antigen; this may facilitate the production of human monoclonal antibodies. Hybridoma cells may be prepared by fusing spleen cells from an immunised animal with a tumour cell. Appropriately secreting hybridoma cells may thereafter be selected. (See Kohler & Milstein *Nature* 1976 296 495).

The invention also provides a suitable technique for purifying HIV antigens. This aspect of the invention is based on the fact that it is generally relatively easy to separate particles from associated impurities (for example by filtration or centrifugation). Therefore, there is also provided a method of producing a substantially pure HIV antigen, the method comprising separating particles as described above from associated impurities and subsequently cleaving HIV antigen from the fusion proteins of the particles.

Fusion protein and particulate antigens of this invention are useful as diagnostic reagents. Particulate antigens are useful as diagnostic reagents. Particulate antigens for diagnostic purposes are particularly advantageous because they can be physically separated by centrifugation or filtration and can be directly dispersed on solid supports such as glass or plastic slides, dip sticks, macro or micro beads, test tubes, wells of microtiter plates and the like. The particulate antigens of this invention may also be dispersed in fibrous or bibulous materials such as absorbent disk (see U.S. Pat. No. 4,632,901), strips or chromatography columns as the solid support. The particles and fusion proteins readily adhere to solid supports. The particles may after purification be disrupted into fusion proteins and the fusion proteins may be dispersed on surfaces as indicated above. These reagents are useful for a variety of diagnostic tests. For example, a test sample suspected of having antibody to the particulate antigen and a fluorescent enzyme or radio-labeled antibody is competitively reacted with the particulate antigen or fusion proteins on a solid support and the amount of labeled antibody which binds to the particulate antigen on the solid support. Particulate antigens of this invention are also useful for agglutination reactions with antibodies. Those skilled in the diagnostic arts will recognize a wide variety of application of particulate antigens and fusion proteins of this invention for diagnostic purposes.

The invention is now illustrated by the following Examples, with reference to the accompanying drawings, in which the letter T followed by a number refers to MD40-4c transformed with a plasmid of that number.

FIG. 1 is a photograph of Ty virus-like particles (Ty-VLPs) purified from MD40-4c transformed with plasmid pMA91-11.

FIG. 2 is a schematic diagram of the construction of pMA5620.

FIG. 3 is a diagram of plasmid pMA5620 with an expanded diagram of the key components of this example of the invention and the nucleotide sequence around the unique BamHI site.

FIG. 4 shows the nucleotide sequences around the BamHI sites of plasmids pMA5620, pMA5621 and pMA5622.

FIG. 5 shows the approximate position of fragments hiv3 to hiv 10 on the map of the *tav* region of HIV.

FIG. 6 shows the nucleotide sequence of the 5' ends of fragments hiv3 and hiv 10.

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FIG. 7 shows a Western blot of sucrose gradient fractions from extracts of MD40-4c and MD40-4c transformed with pMA5620-hiv5 and pMA5620-8. The blotted proteins are probed with anti-Ty-VLP antibody.

FIG. 8 shows an electron micrograph of hybrid Ty-HIV-VLPs produced from pMA5620-hiv5.

FIG. 9 is a diagram of the approximate position of fragment hiv22 on the map of the gag region of HIV and the nucleotide sequence and amino acid sequence at the TYA-hiv22 junction in the plasmid pMA5620-hiv22.

FIG. 10 is the nucleic acid sequence and deduced amino acid sequence of the p1:p24 fusion protein encoded by the plasmid pMA5620-hiv22.

FIG. 11 is the sequence of two synthetic HIV oligomers.

FIG. 12 is the nucleic acid sequence and deduced amino acid sequence of the p1:hiv31 fusion protein encoded by the plasmid pMA5620-hiv31.

FIG. 13 is the nucleic acid sequence and deduced amino acid sequence of the p1:hiv32 fusion protein encoded by the plasmid pMA5620-hiv32.

The amino acid symbols in the drawings are as follows:

The amino acid symbols in the drawings are as follows:		
Amino Acid	One-letter Symbol	
Alanine	A	
Arginine	R	
Asparagine	N	
Aspartic acid	D	
Asn and/or Asp	B	
Cysteine	C	
Glutamine	Q	
Glutamic acid	E	
Gln and/or Glu	Z	
Glycine	G	
Histidine	H	
Isoleucine	I	
Leucine	L	
Lysine	K	
Methionine	M	
Phenylalanine	F	
Proline	P	
Serine	S	
Threonine	T	
Tryptophan	W	
Tyrosine	Y	
Valine	V	

EXAMPLE 1

Strains used were *E. coli* AKEC28 (C600, thyC, thrA, trpC1117, hsdRK, hsdK) and *S. cerevisiae* MD40-4c (urd2, trt1, leu2-3, leu2-112, his3-11, his3-15). *E. coli* media were prepared according to Miller (Miller 1972 Experiments in Molecular Genetics, CSH p433) and yeast media were prepared according to Hawthorne and Mortimer (Hawthorne and Mortimer 1960 Genetics 45, 1085).

E. coli was transformed using standard methods (Maniatis et al. 1982 Molecular cloning - A Laboratory Manual, CSH p199). Yeast was transformed as described by Hinnen et al. (Hinnen et al. 1978 Proc. Natl. Acad. Sci. 75, 1929).

Standard procedures were used for restriction digestion and plasmid constructions (Maniatis et al. 1982 op. cit). Restriction enzymes and T4 DNA ligase were used according to the suppliers instructions. Bal 31 exonuclease digestions were carried out as described by Dobson et al. (Dobson et al. 1982 Nucl. Acids Res. 10, 5463).

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Deletion end points were determined by DNA sequencing (Sanger et al. 1977 Proc. Natl. Acad. Sci. 74, 5463). BamHI synthetic oligonucleotide linkers were obtained from Pharmacia.

Plasmid DNA was isolated from *E. coli* preparatively as described by Chinault and Carbon (Chinault and Carbon 1979 Gene 5, 111) and for rapid analysis by the method of Holmes and Quigley (Holmes and Quigley 1981 Anal. Biochem. 114, 193).

Ty-VLPs were purified as follows. Yeast cells were grown selectively at 30° C. to a density of 8×10^6 cells/ml⁻¹. The cells were then collected by low speed centrifugation, washed once in ice-cold water and resuspended in TEN buffer (70 mM Tris, pH 7.4; 2 mM EDTA; 140 mM NaCl) at 1 ml per 1 liter of cells. The cells were disrupted by vortexing with glass beads (40-mesh; BDH) at 4° C. until >70% were broken. The beads were pelleted by low speed centrifugation, then the supernatant was collected and the debris removed by centrifugation in a microfuge for 20 minutes. The Ty-VLPs were then pelleted from the supernatant by centrifugation at 100,000 g for 1 hour at 4° C. and by resuspending overnight in TEN buffer. The resuspended Ty-VLPs were centrifuged in a microfuge for 15 minutes at 4° C. to remove cell debris prior to loading the supernatant onto a 15-45% (w/v) sucrose gradient in 10 mM Tris, pH 7.4; 10 mM NaCl and spinning at 76,330 g for 3 hours at 15° C. Fractions were collected through the bottom of the tube and the peak fractions were identified by running aliquots of the fractions on SDS-PAGE gels and Coomassie blue staining. VLPs were concentrated by centrifugation of the peak fractions at 100,000 g for 1 hour at 4° C.

Protein extracts of whole yeast cells were prepared as previously described (Mellor et al. 1983 Gene 24, 1). Gel procedures were those of Laemmli (Laemmli 1970 Nature 227, 68). Protein concentrations were measured by a dye-binding assay (Bradford 1976 Anal. Biochem. 72, 248) obtained from Bio-Rad Laboratories.

Plasmid pMA91-11 has been described previously (Dobson et al. 1984 EMBO J. 3, 1115): it contains the first 1450 nucleotides of the major transcriptional unit of the Ty element, Ty1-15, inserted into the high efficiency expression vector pMA91 (Mellor et al. 1983 op. cit.; Kingman and Kingman 1985 Biotech. and Genet. Eng. Rev. 3, 377). The Ty component was derived from pKT40b as described by Dolson et al. (op. cit): pKT40b has been deposited with the National Collection of Industrial Bacteria, Aberdeen, U.K. under accession number NCIB 12427. In turn, the expression vector pMA91 consists of plasmid pBR22 sequences which allow replication and selection in *E. coli*, the yeast 2 micron plasmid origin of replication, which allows efficient autonomous replication in yeast, the yeast LEU2 gene as a selectable marker in both yeast leu2 and *E. coli* leuB mutants and a Bg/II expression site which separates the upstream non-coding region of the yeast PGK gene from -1500 to -1 from the 3' region of PGK which contains all the signals for yeast transcription termination. Plasmid pMA91 is also described in U.S. Pat. No. 4,615,974, although it should be carefully noted that the plasmid designated as pMA3013 in FIG. 15 of this U.S. Patent is what is now known as plasmid pMA91. The plasmid shown in the lower part of FIG. 1 of U.S. Pat. No. 4,615,974 has since been renamed.

Ty expression is driven, therefore, from the promoter of the highly efficient yeast phosphoglycerate kinase gene (PGK) and yeast extracts of strains containing

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pMA91-11 overproduce massive amounts of p1 protein, the primary translation product of the TYA gene (Dobson et al. 1984 op. cit; Mellor et al. 1985a op. cit). We now demonstrate that extracts of yeast transformants containing pMA91-11 contain Ty-VLPs in large quantities (FIG. 1). Therefore, TYA alone contains sufficient information to make TyOVLPs and the p1 protein found in extracts of MD40-4c containing pMA91-11 is assembled into particles (FIG. 1).

The construction of a plasmid vector, pMA5620, that would direct the synthesis of any hybrid Ty-VLP particle is shown schematically in FIG. 2. This required the construction of a vector containing a convenient restriction endonuclease site within the TYA gene such that any coding sequence can be inserted into that site to create a TYA hybrid gene. However, it is essential that within such a hybrid there is sufficient TYA coding sequence to direct the synthesis of Ty-VLPs.

Plasmid pMA91-11 was cleaved with BglII, digested with Bal 31 exonuclease for various times and re-ligated in the presence of excess Bam HI linkers (CCGGATCCGG). The deletion end points of the resulting plasmids were determined by DNA sequencing. Plasmid pMA91-357 is a deletion derivative in which 265bp have been removed. This places the BamHI linker one nucleotide beyond codon 381 of TYA (FIG. 3).

In order to provide both transcription termination sequences and translation stop codons in all three reading frames the deleted PGK 3' terminator sequences of pMA91-357 were replaced with a 287 bp BamHI-SalI DNA fragment isolated from plasmid pDT86. This DNA fragment is a modified 3' transcription terminator fragment from the yeast PGK gene which contains translation stop codons in all three reading frames downstream of the BamHI site (FIG. 2). This terminator fragment starts with a BamHI linker (CCGGATCCGG) linked to the last sense codon of the PGK coding sequence and extends to the HindIII site 279 nucleotides beyond the PGK coding sequence (Hitzeman et al. 1982 Nucl. Acids Res. 10, 7791). In these constructions the HindIII site has been converted to a SalI site using synthetic linker. The terminator fragment is not critical and any fragment containing termination codons in all three reading frames followed by a yeast transcription terminator would suffice. The resulting plasmid, pMA5620, contains a unique BamHI site into which any suitable sequence can be inserted to produce a hybrid protein which will be assembled into hybrid Ty-VLPs.

Plasmid pMA5620 is shown in FIGS. 2 and 3. It can accept the coding sequence of any antigen at its unique BamHI site and then direct the synthesis in yeast of the resulting fusion protein. Clearly the antigen coding sequence must be inserted in such a way that it is in the same translational reading phase as TYA. In order to simplify this for any restriction fragment containing an antigen coding sequence two derivatives, pMA5621 and pMA5622, of pMA5620 were constructed in which the cleavage points of their unique BamHI sites placed at positions that correspond to TYA translational reading phases +1 and +2. pMA5621 and pMA5622 were constructed by cleaving pMA5620 with BamHI, filling in the 5' extensions with DNA polymerase I and then ligating in the presence of oligonucleotide AAG-GATCC for pMA5621, and oligonucleotide GGATCC for pMA5622. The sequence of the constructions was confirmed by dideoxynucleotide se-

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quencing, FIG. 4 shows a comparison of the sequences of pMA5620, pMA5621 and pMA5622 around their unique BamHI sites with respect to the reading phase of TYA.

In order to produce hybrid Ty:HIV-VLPs it was necessary to insert fragments of the HIV proviral genome into pMA5620, pMA5621 and pMA5622. HIV DNA was from the proviral form of viral isolate HIV 1b (HTLV IIb) as reported by Ratner et al. (1985 Nature 313, 277). Plasmid pHIVX is plasmid pSP46 (Promega Biotec) containing a 8931 bp SstI fragment that starts at the second SstI site of the provirus and ends at the third. This fragment contains therefore all of the viral coding regions and was the source of the HIV fragments.

Eight HIV fragments from the env coding region of the virus were chosen for an initial study. These were designated fragments hiv3, hiv4, hiv5, hiv6, hiv7, hiv8, hiv9 and hiv10. Fragment hiv3 is a KpnI:PvuII fragment corresponding approximately to codons 41 to 287; hiv4 is a DraI:HindIII fragment corresponding approximately to codons 341 to 639; hiv5 is a DraI:DraI fragment corresponding approximately to codons 129 to 341; hiv6 is a Sau3a:Sau3a fragment corresponding approximately to codons 25 to 112; hiv7 is a Sau3a:Sau3a fragment corresponding approximately to codons 112 to 272; hiv8 is a Sau3a:Sau3a fragment corresponding approximately to codons 272 to 466; hiv9 is a Sau3a:Sau3a fragment corresponding approximately to codons 466 to 588; hiv10 is a Sau3a:Sau3a fragment corresponding approximately to codons 588 to 743.

FIG. 5 shows the approximate positions of these fragments on a map of the HIV env region. Each of the fragments were purified from agarose gels and inserted into either pMA5620, pMA5621 or pMA5622 by various means: hiv8 was inserted via a sticky-end ligation into pMA5620 to produce pMA5620-hiv8; hiv6 and hiv9 were inserted into pMA5621 via sticky-end ligations to produce pMA5621-hiv6 and pMA5621-hiv9 respectively; hiv7 and hiv10 were inserted into pMA5622 via sticky-end ligations; hiv4 and hiv5 sticky-ends were filled in by DNA polymerase I and then the fragments were blunt-end ligated into pMA5620 that had been cleaved at the BamHI site and also filled in to produce pMA5620-hiv4 and pMA5620-hiv5; hiv3 sticky-ends were filled in and then the fragment was blunt-end ligated into pMA5621 that had been cleaved at the BamHI site and also filled in to produce pMA5621-hiv3. FIG. 6 shows the nucleotide sequence of the 5' ends of the hiv3-10 fragments.

Each of the plasmids was used to transform yeast strain MD40-4c to leucine independence. Extracts of the resulting transformants were then analysed for the presence of hybrid Ty:HIV-VLPs. VLPs were prepared from transformants and fractions from a 15-45% sucrose gradient were run on an SDS-PAGE gel. The proteins were visualised by Western blotting and then probed with an anti-Ty-VLP antibody. FIG. 7 shows the results for pMA5620-hiv5.

The fusion of the part of TYA present in pMA5620 to hiv5 should produce a new Ty:HIV protein of about 70 kd. This protein, if it is in particulate form, should co-migrate with the particles in extracts of MD40-4c transformed with pMA5620-hiv5 but contains an interferon-alpha2 cDNA instead of an hiv5 fragment (UK patent application No. 8626148). The data in FIG. 7 show that this is the case suggesting that pMA5620-hiv5 directs the production of hybrid Ty:HIV-VLPs. Similar results

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are obtained with the other constructions. The fractions of the sucrose gradient that contained the 70 kd fusion protein were examined by electron microscopy and were shown to contain particles (FIG. 8). We would conclude therefore that pMA5620-hiv5 directs the production of hybrid Ty:HIV-VLPs.

In order to establish that these VLPs do in fact carry HIV epitopes fractions containing particles from the sucrose gradients may be pooled and samples were run on an SDS-PAGE, electroblotted onto nitrocellulose and then probed with anti-HIV antiserum. As a positive control for this experiment disrupted HIV may be run alongside the particle proteins.

EXAMPLE 2

In order to test the efficacy of the hybrid Ty:HIV-VLPs produced as in Example 1 in eliciting an immune response to the HIV component antisera are raised in rabbits against concentrated hybrid Ty:HIV-VLPs purified from MD40-4c transformed with pMA5620-hiv5 and pMA5620-hiv8. These antisera are then used in a Western blot against disrupted HIV, from which it may be seen that they react showing that the hybrid Ty:HIV-VLPs induce the production of anti-HIV antibodies.

It is important that an antigen that is to be used as a vaccine induces the production of neutralising antibodies. To test this for the hybrid Ty:HIV-VLPs the antisera are tested in an HIV infectivity neutralisation assay, from which it may be seen that the antisera will neutralise HIV showing that the hybrid Ty:HIV-VLPs are useful as a vaccine.

These data show: (1) fusion proteins composed of 381 amino acids of the TYA gene and various fragments from HIV are readily produced; (2) these fusion proteins produce polyvalent hybrid Ty:HIV-VLPs; (3) these hybrid Ty:HIV-VLPs react with anti-HIV antiserum; (4) the hybrid Ty:HIV-VLPs induce the production of neutralising anti-HIV antibodies in rabbits. It is reasonable therefore to expect that pMA5620, pMA5621 or pMA5622 will direct the expression of polyvalent hybrid Ty:HIV-VLPs containing any HIV antigen from gag, pol, env, sor, tat, art/trs or 3'ort. Any of these antigens may constitute a key component in a vaccine or a diagnostic kit.

EXAMPLE 3

The Production of Hybrid HIV:TY-VLPs Carrying p24 Antigens

The HIV gag precursor protein is cleaved into three mature proteins p17, p24 and p15, that form the virus core. The p24 protein is particularly significant from a diagnostics point of view as HIV positive asymptomatic individuals have high titres of anti-p24 antibody. Furthermore, p24 may be significant in the production of a vaccine (Salk, 1987, Nature 327, 473).

In order to construct hybrid HIV:Ty-VLPs carrying p24 antigens, a HindIII fragment, hiv22, from the gag gene of the HIV proviral genome was inserted in the BamHI site of pMA5620. hiv22 starts at nucleotide 1082 and ends at 1709 (Ratner et al; 1985 Nature 313, 277) and it encodes amino acids 99 to 308 of gag. This region contains, therefore, the carboxy-end of p17 and most of p24 (FIG. 9). hiv22 was inserted into pMA5620 such that the TYA and gag sequences were in frame (FIG. 9) and the resulting molecule was designated pMA5620-hiv22. This plasmid was introduced into yeast strain

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MD40-4c and extracts of the transformants were analysed on 15-45% sucrose gradients. Coomassie stained SDS-PAGE gels of the gradient fractions revealed the presence of a fusion protein of about 75 kD, the expected size of a p1:p24 fusion protein, in the region of the gradient characteristic of particulate structures. That this protein carries p24 antigens was confirmed by blotting a similar gel and probing with a p24 monoclonal antibody. This showed a clear peak of reacting material well down the gradient at a position diagnostic of particulate structures. The particulate nature of the p1:p24 fusion was further confirmed by examining peak fractions in the electron microscope. Numerous particles were seen. FIG. 10 shows the amino acid sequence and nucleotide coding for p1-p24.

These data show that hybrid HIV:Ty-VLPs carrying p24 antigens can be produced and that the antigen reacts with an anti-p24 monoclonal antibody. This system may be used to produce p24 antigens for diagnostics or an anti-HIV vaccine.

EXAMPLE 4

Synthetic Oligonucleotides to Produce Ty:HIV Fusion Genes

The oligonucleotides were synthesized by automated phosphoramidite chemistry using cyanoethyl phosphoramidites (Beaucage and Caruthers 1981 Tetrahedron Letters 24, 245). Following de-blocking and removal from the controlled pore glass support the oligomers were purified on denaturing polyacrylamide gels, further purified by ethanol precipitation and finally dissolved in water prior to estimation of their concentration. The oligomers were then kinased to provide them with a 5' phosphate as required for the ligation step. Complementary oligomers were then annealed prior to ligation into the relevant plasmid vector. The sequence of the synthetic oligomers was confirmed by dideoxy sequencing. The protocol used was essentially as has been described (Biggin et al. 1983 Proc. Natl. Acad. Sci. 80 m 3963) and modified to allow sequencing on plasmid DNA as described (Guo and Wu 1983 Nucl. Acids Res. 11, 5521).

In order to construct hybrid HIV:Ty-VLPs carrying env antigens two DNA oligomers were synthesized. Oligomer hiv31 is 105 bp in length and encodes amino acids 495 to 527 of the env precursor protein gp160 of HIV 1b (HTLVIIIb; Ratner et al. 1985 Nature 313 277): it spans the cleavage site between gp120 and gp41. Oligomer hiv32 is 114 bp in length and encodes amino acids 53 to 88 of gp120 of HIV 1b (HTLVIIIb; Ratner et al. 1985 Nature 313, 277). The codons were selected to be those that are favored by yeast (Maruyama et al. Nucl. Acids Res. Suppl. 14, 151). The end points of the oligomers were designed such that following ligation into pMA5620 cleaved with BamHI, a BamHI site would be re-created at the 3' end of the insertion and a BamHI/BglII junction generated at the 5' end (FIG. 10).

The synthetic oligomers hiv31 and hiv32 (FIG. 11) were ligated into pMA5620 that had been cleaved at the BamHI site. The resulting plasmids are designated pMA5620-hiv31 and pMA5620-hiv32. The complete nucleotide sequence and the deduced amino acid sequence of the HIV:Ty fusion proteins encoded by these two plasmids is shown in FIGS. 12 and 13.

Plasmids pMA5620-hiv31 and pMA5620-hiv32 were used to transform yeast strain MD40-4c to leucine independence. Extracts of the resulting transformants were

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then analysed for the presence of hybrid HIV:Ty-VLPs. VLPs were prepared from the transformants and fractions from a 15-45% sucrose gradient were run on SDS-PAGE gels. In both cases Coomassie blue staining revealed the presence of a fusion protein of about 54 kD, the expected size of both of the HIV:Ty fusion proteins, in the region of the gradient characteristic of particulate structures, although it is noticeable that the precise position differs for the two types of particles.

These data show that synthesized oligomers can be used in the construction of hybrid HIV:Ty fusion genes. Overexpression of these genes results in the production of hybrid HIV:Ty-VLPs. This system could be used to produce defined env antigens for diagnostics or an anti-HIV vaccine.

EXAMPLE 5

Enzyme Immunoassay Procedure with VLP Having an HIV Antigen

96-well microtitre plates are coated with VLP having a fused HIV antigen (VLP-HIV) by incubating 50 µl of 20 µg/ml of VLPs in 50 mM sodium carbonate buffer, pH 9.5, in each well for two hours at room temperature. Excess VLPs HIV are washed out of the wells by three, five minute washes with phosphate buffered saline (PBS), pH 7.4. In order to minimize background reactions, the wells are blocked with 100 µl of 2% casein in PBS for one hour at room temperature, followed by three, five minute washes with PBS containing 0.1% Tween-20 (PBS-T). A test sample is suitably diluted in PBS-T containing 0.5% casein (PBS-CT). A suitable dilution may be a three-fold dilution series from 1/10 to 1/7290. The HIV antibody in the test sample is reactive to the HIV component of any hybrid VLP-HIV. 50 µl of diluted test sample antibody is added to the appropriate wells and incubated for two hours at room temperature. Excess test sample antibody is removed by three, five minute washes with PBS-T. Secondary antibody is horseradish peroxidase-labeled anti-species IgG, and is diluted 1/1,500 in PBS-CT. 50 µl of diluted secondary antibody is added to each well and incubated for two hours at room temperature, followed by five, five minute washes with PBS-T. The substrate is 3,3',5,5'-tetramethylbenzidine at a concentration of 0.1 mg/ml in 0.1 M sodium acetate, adjusted to pH 6.0 with 0.5 M citric acid, plus 0.03% hydrogen peroxide. 50 µl of substrate is added to each well and the color reaction developed for 10 minutes. The reaction is terminated by the addition of 25 µl of 0.5 M sulfuric acid to each well. Color development is assessed by measurement at 450 nm using a microplate reader. In this way a direct assay of the HIV antibody in the test sample is performed.

What is claimed is:

1. A particle comprising a plurality of fusion proteins, each fusion protein comprising a first amino acid sequence and a second amino acid sequence, wherein the first amino acid sequence is substantially homologous with a particle-forming protein encoded by a yeast TYA gene without TYB gene sequence and wherein the second amino acid sequence is substantially homologous to an HIV antigen.
2. A diagnostic reagent comprising particles of claim 1 dispersed on a solid support.
3. A particle according to claim 1 wherein the second amino acid sequence is immunologically reactive to antibodies which are immunologically reactive to HIV proteins p24, p41 or p120.